Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Herpesvirus of Turkeys (Strain FC-126), Lyophilized

Date: October 29, 1998

Supersedes: April 15, 1974

Number: PYSAM0407.01

Standard Requirement: 9 CFR 113.330

Contact Person: Rebecca L. W. Hyde, (515) 239-8336

Approvals:

_____ Date:_____
Rebecca L. W. Hyde, Head
Poultry Biologics Virology Section

_____ Date:_____
Richard E. Hill, Quality Assurance Manager

_____ Date:_____
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for Titration of Herpesvirus of Turkeys (Strain FC-126), Lyophilized

Table of Contents

Supplemental Assay Method for Titration of Herpesvirus of Turkeys (Strain FC-126), Lyophilized

1. Introduction

This Supplemental Assay Method (SAM) describes a procedure using chick embryo fibroblast (CEF) cell cultures, for titrating cell free herpesvirus of turkeys (Strain FC-126) used as a vaccine against Marek's disease. The vaccine is composed of lyophilized cell-free preparation of the virus and a suitable diluent.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)

2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model 252)

2.1.3 Water-jacketed incubator with a humidified 5% CO_2 atmosphere and temperature set at 37°C (Forma Scientific, Model No. 3158)

2.1.4 Vortex mixer (Thermolyne Maxi Mix II Model No. M37615)

2.1.5 Magnetic stir plate

2.1.6 Scissors, sterile (Roboz RS-6800)

2.1.7 Curved tip forceps, sterile (V. Mueller SU 2315)

2.1.8 Pipette (Rainin Pipetman, P1000, or equivalent)

2.1.9 250-ml trypsinizing flask with stir bar, sterile

2.2.10 Erlenmeyer flask with a stirring bar, sterile.

2.1.11 Hemocytometer

2.1.12 Bunsen burner

2.1.13 Blunt thumb forceps, sterile

2.2 Reagents/supplies

Supplemental Assay Method for Titration of Herpesvirus of Turkeys (Strain FC-126), Lyophilized

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Cotton swab

2.2.2 Tissue culture dish, 150x10 mm (Falcon, Cat. No. 1058)

2.2.3 Tissue culture dish, 100x20 mm (Falcon, Cat. No. 3003)

2.2.4 Plastic funnel covered with 4 layers of fine gauze

2.2.5 Polypropylene conical tube, 29x114 mm, sterile, 50 ml (Sarstedt, Cat. No. 62.547.205)

2.2.6 Polypropylene centrifuge tubes, 250 ml (Corning, Cat. No. 25350)

2.2.7 Roller bottles, 1000 ml (plastic)

2.2.8 Serological pipets (Falcon, Cat. No. 7530)

2.2.9 60-mm gridded cell culture dish, tissue culture treated (Costar, Cat. No. 3160)

2.2.10 2 dozen specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old

2.2.11 Fetal Bovine Serum (FBS)

2.2.12 L-Glutamine (Sigma, Cat. No. G7513)

2.2.13 Trypsin, 0.25% (Cello Corporation, Cat. No. AT25)

2.2.14 Pipette tips (Rainin 0-100, 0-200, 100-1000 or equivalent)

Supplemental Assay Method for Titration of Herpesvirus of Turkeys (Strain FC-126), Lyophilized

2.2.15 Solutions

All solutions are filter sterilized.

1. Trypsin Solution (0.25%):

NaC1	8.0 g
Kcl	0.4 g
Glucose	1.0 g
Phenol Red (0.5% solution)	1.0 ml
Trypsin (1:250)	2.5 g
$NaHCO_3$	0.35 g
q.s. with distilled or deionized water	1.0 L

Adjust pH to 7.4 with $NaHCO_3$ solution.

2. Growth Medium:

Medium 199 (with Earle's salts)(powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth (dry powder)	2.95 g
$NaHCO_3$	2.5 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
Fetal Bovine Serum*(heat inactivated)	85 ml
q.s. with distilled or deionized water	2185 ml

Adjust pH to 7.35 to 7.4 by adding $NaHCO_3$ solution. Before use, add 1.0 ml of a 200 mM concentration of L-glutamine per 100 ml medium.

3. Maintenance Medium:

Medium 199 (with Earle's salts)(powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth(dry powder)	2.95 g
$NaHCO_3$	2.75 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
Fetal Bovine Serum* (heat inactivated)	42 ml
q.s. with distilled or deionized water	2142 ml

Adjust pH to 7.5 by adding $NaHCO_3$ solution. Before use, add 1.0 ml of a 200-mM concentration of L-glutamine per 100 ml medium.

*Previously tested for freedom from extraneous agents

4. SPGA Diluent:

Sucrose	74.62 g
KH_2PO_4	0.45 g
K_2HP0_4	1.35 g
Mono Sodium Glutamate	0.80 g
1% Bovine Albumin (Fraction V)	10.0 g
q.s. with distilled or deionized water	1000 ml

2.2.16 Cell cultures

Primary chick embryo cell cultures are used for the titration.

Prepare primary chick embryo cell cultures from 9- to 11-day-old chick embryos (derived from specific-pathogen-free flocks) in the following manner: Swab the air cell end of the egg with 70% ethanol, flame, and break open the shell with sterile blunt thumb forceps. Use the forceps to open the membranes, lift out the embryo, and place it in a sterile disposable petri dish. Four to six embryos may be prepared together. Remove (and discard) the heads of the embryos with sterile scissors. Wash the embryos by adding 0.25% trypsin solution to the petri dish. Open the body cavity of the embryos with the sterile forceps and remove the liver and the bulk of the other viscera. Gently squeeze the remainder of the embryos with the forceps to remove as much blood as possible. Pick the washed embryos out of the wash solution with the forceps, drain them momentarily, and place them in a sterile, dry petri dish. Mince the embryos thoroughly by cutting with sharp, sterile scissors.

Place the minced tissue in a 250-ml sterile trypsinizing flask with a magnetic stirring bar, add 30 ml of 0.25% trypsin solution (prewarmed to approximately 35°C), and trypsinize for 20 min at room temperature. Carefully decant the supernatant suspension through a sterile funnel with 4 layers of gauze into a sterile centrifuge bottle.

Add approximately 30 ml of growth medium (see part **2.2.15**) to the centrifuge bottle to stop the action of the trypsin on the cells. To the remaining fragments in the trypsinizing flask, add another 30 ml of 0.25% trypsin and repeat the process for another 20 min. Add this cell suspension to the first collection. Centrifuge at approximately 250 X g for 10 min. Remove the supernatant fluids carefully. Resuspend the cells in growth medium to a concentration

of approximately 750,000 cells per ml. Plant the cells in 60-mm plastic petri dishes (gridded or plain), 5 ml per dish (approximately 3.75×10^6 cells). Incubate in a high-humidity atmosphere containing approximately 5% CO_2). In 24 hr the monolayers should be complete and ready for inoculation.

3. Preparation for the test

3.1 Personnel qualifications/training

The executor must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. The executor must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent; and training in the operation of the necessary laboratory equipment listed in part **2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturer’s instructions and monitor in compliance with current corresponding CVB-L/National Veterinary Services Laboratories Standard Operating Procedures (SOPs), or equivalent.

3.3 Preparation of reagents/control procedures

Prepare reference viruses in the same manner as sample preparation.

Supplemental Assay Method for Titration of Herpesvirus of Turkeys (Strain FC-126), Lyophilized

3.4 Preparation of the sample

3.4.1 Preparation of vaccine for titration

Rehydrate the vaccine (HVT) to field strength with the proper amount of the manufacturer's diluent. Mix thoroughly.

3.4.2 Holding period

Place the vaccine bottle in an ice bath for 2 hr prior to proceeding with the titration.

4. Performance of the test

4.1 Preparing dilutions and inoculating plates

Prepare dilution blanks of 9 ml using the manufacturer's diluent or SPGA diluent. Remove the medium from the titration plates in this manner: Set the plates on a slanted surface. Remove fluid with a drawing off apparatus, allow the plates to drain an additional 60-90 sec, and remove the accumulated fluid at the lower edge of the plates. Remove the vaccine from the ice bath and invert several times to mix the virus suspension thoroughly. Remove a 1.0-ml representative sample from the vaccine and add to the 9-ml dilution blank. Mix thoroughly. This makes a 1:10 dilution of the vaccine (for vaccine expected to have a relatively high-virus content, a 1:20 dilution may be used). Mix this dilution thoroughly, and inoculate 0.05 ml per plate onto the center of each of 5 plates (on a horizontal surface). Since the bottoms of most plastic plates are slightly convex, the inoculum will usually spread without tipping the plates. Incubate the inoculated plates at 37.5°C in a humidified atmosphere for a 30-45 min absorption period. Add 5 ml maintenance medium to each plate, and continue incubation at 37.5°C in a high-humidity atmosphere of approximately 5% CO_2 for 6 days. The maintenance medium may be replaced after 2 or 3 days if the pH of the culture fluids becomes too acidic.

Supplemental Assay Method for Titration of Herpesvirus of Turkeys (Strain FC-126), Lyophilized

5. Interpretation of the test results

5.1 Controls

Titrate a known positive reference virus with each group of titrations. The titer of the positive reference must be within the established range for the test results to be valid. Uninoculated negative control cells are maintained to monitor the integrity of the cell culture system.

5.2 Making foci counts and calculating titer

5.2.1 Counting

1. Well-developed foci usually may be observed by day 4 postinoculation (PI). On day 6 PI, make a foci count using an inverted microscope (and a grid-adapted stage if plain plates have been used). By day 6, all primary foci should have developed and secondary foci should not be developed well enough to cause problems in counting using these testing methods.

2. Count all the foci on each of the plates of the titration series. A focus is counted as 1 regardless of size unless it has apparently arisen from 2 distinct centers. At least 4 plates should be counted for a valid test.

5.2.2 Calculating focus-forming units (FFU)

Calculate the average number of foci per plate and multiply this value by 40 (or 80)*. This result will be the focus forming units (FFUs) per bird dose (assuming the vol of 1 bird dose is 0.2 ml).

5.3 Retests

Conduct retests as required by the Code of Federal Regulations, Title 9, part 9 113.8 (b) and requirements of minimum release in firm’s current Outline of Production, Part V.

---

*10 (dilution factor) times 4 (inoculum is equivalent to ¼ bird dose) equals 40; or 20 (dilution factor) times 4 equals 80.

5.4 Evaluation of test results

5.4.1 The 9CFR 113.8 (b) defines the criteria for a satisfactory/unsatisfactory serial.

5.4.2 The firm's requirements of minimum release/stability titers for each Marek's vaccine are listed in the current Outline of Production, Part V, for the specific product code.

6. Report of test results

Titers are reported out as FFUs per bird dose.

7. References

This document was rewritten to meet the current CVB-L QA SAM format. No significant changes were made from the previous protocol. This document supersedes the April 15, 1974, version.